Immunoevaluation and Characterization of Tetanus Toxoid by using Natural polymer as an adjuvant

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Abstract : Natural polymer like potato starch is a mixture of amylose and amylopectin, . Extraction of Potato starch was performed by rasping, centrifugation, refining and drying method. In our research method, we employed potato starch as a biodegradable polymer; It has a great impact on pharmaceutical applications due to its bioavailability, non toxic, high change density and biodegradability. In our research work, we have selected Potato starch polymer as a model of immunomodulatory effect of vaccine of tetanus antigen. According to WHO, Tetanus is a systemic infectious disease caused by genus *Cl Tetanii.* It has been estimated that the tetanus fever endemicity among large populations and global emergence of multidrug resistant strains to impose greater urgency on the evaluation of existing and new vaccines to prevent mortality of neonates and in pregnancy. Recently available recombinant vaccine was seems to be side effect and cost effective. The starch polymer in the form of microspheres was preferred in order to replace the alum to elicit sustained immune response because alum induces local granuloma and hypersensitivity reaction to some individual. We have employed microencapsulation technique by using 0.5% ml glutaraldehyde as a crosslinking agent. The particle size was analyzed as 40.23µm. *Invitro* studies was analyzed by SEM, stabilities studies Immunogenicity studies was carried out by incubating the sample, centrifuged and tested for an antigen and the Compatibility study was performed by Infrared Spectroscopy, the antigen integrity was studied by SDS PAGE and ELISA. Immunoglobulin titer values was found out (IgG, IgA,IgM, IgE) to show the increase level of antibody response.

Key-words: Natural polymer, Tetanus antigen, Immunological evaluation, Antibody

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INTRODUCTION: Tetanus is more common in the developing countries, where the climate is warm and in rural areas, poor hygienic practices in India. It was a common cause of death particularly in the newborn and mother in pregnancy. The world spread use of vaccines over the last few decades has resulted in a reduction in the incidence of many infectious diseases in developed countries. Nevertheless, there are still significant challenges for vaccine developments including the need to make improvement in existing vaccine by making them safer, more immunogenic and to extend vaccine coverage in certain populations particularly in the developed world[1]. One of the most important issues in vaccinology is the need for new adjuvant in vaccine delivery system[2] Most of the vaccines currently in development are based on purified subunits, recombinant molecule, synthetic peptides or nucleic acids which are often poorly immunogenic, expensive and produce adverse effect[3] It is clear that new generation of vaccines will require better adjuvant delivery systems to induce optimal immune response. The system under discussion employ either biodegradable polymer or system requiring removal after use and can release the drug either by membrane or matrix- controlled diffusion. Recent trends in potential carrier delivery have seen microencapsulation of pharmaceutical substances in biodegradable polymers as an emerging technology[4]. Currently,Biodegradable polymer in the form of microsphere has shown the ideal perquisite for microsphere carrier in vaccine delivery system[5,6]

The enhanced immunogenicity of particulates antigens is unsurprising, since pathogens are particulates of similar dimensions and the immune system has evolved to deal with these Particulates delivery system[7] It presents multiple copies of antigens to the immune system and promote

trapping and retention of antigen in local lymph node moreover particles are taken up by macrophages and dendrite cells leading to enhanced antigen penetration and the release of cytokines to promote the induction of an immune response Sustaining response for a longer duration including local mucosal immune response, generating antibody with increased avidity and neutralization capacity eliciting cytotoxic T lymphocyte (CTLs) to enhance immune response.[8]

Potato Starch can be modified through physical, chemical or enzymatic processes[9] and used as food additives because of their considering safety and low cost. Very low levels are used and these are approved by the FDA (CFR 172.892)[10.]

MATERIAL AND METHODS: sample of Typhoid antigen obtained from Serum Institute of India Guindy (Chennai), Organic solvent of AR. grade, Tween-80 (hi-media), Glutaraldehyde (spectrochemicals). *Starch* (chemical formula (C6H10O5) n,) is a mixture of *amylose* and *amylopectin*. The former consists of long, unbranched chains of D-glucose residues connected by (α1→4) linkages. Potato was washed efficiently to remove dirt, fungi, rotten spots. Rasping was performed to release the tuber cells and Starch. The potato juice is generally rich in sugar and protein. So when opening the cells the juice is instantly exposed to air and reacts with the oxygen, forming coloured components, which may adhere to the starch. This can be reduced by adding sufficient amount of sulpurdioxide gas to maintain the juice and pulp light yellow. The sample was centrifuged at 5,000rpm and supernatant sample was collected, dried, and stored.

Tetanus vaccine encapsulated Potato starch microparticles were prepared by microencapsulation techniques (Emulsion crosslinking) Based upon the result obtained by changing various parameters such as effect of polymer concentration and effect of crosslinking agent concentrations various batches were prepared, to standardize an ideal batch. Starch solution (1% to 10% / ml) was prepared in a 250 ml beaker by continuous stirring for one hour at 5000 rpm. An emulsion was prepared by mixing the vegetable oil and toluene with two drops of Tween-80. To the gel 1ml of Tetanus antigen and 1 ml of 0.5% Glutaraldehyde was added and stirring is continued for one hour. Add 2 ml of gel containing tetanus antigen in to the emulsion with continuous stirring. At the end the precipitates were centrifuged, washed with various organic solvents' A white powder were obtained (microspheres) many batches were formulated, pooled and stored in a refrigerator at 4^0C.

Characterization of micro particles: The morphology and size distribution of dried microspheres was evaluated by Scanning Electron Microscopy(SEM) and the size of the microparticles was determined by optical microscope using calibrated scale. The magnitude of loading Tetanus antigen in microparticles was performed by mixing with phosphate buffer saline (pH7.4). Under shaking at room temperature and kept for 3 hrs. The suspension was centrifuged at 4000 rpm for 15 min to remove free antigen. This process was analyzed by Lowry' method.[11]

$$\text{Loading capacity \%} = \frac{\text{Total amount of antigen} - \text{Free antigen}}{\text{Weight of micro particles}} \times 100$$

$$\text{Loading efficiency \%} = \frac{\text{Total amount of antigen} - \text{Free antigen}}{\text{Total amount of antigen}} \times 100$$

The stability of the formulation Tetanus antigen encapsulated starch microparticles and unloaded starch microparticles were determined over period of weeks. Both antigens loaded and unloaded micro particles were kept at 4^0C. At predetermined time intervals the samples were taken at 0, 1, 2, 3, 4, 5, 6, and 7 days. The morphology was determined by light microscope and the size was determined by using stage-eyepiece micrometer.

The swelling ratio of the starch microparticles was determined as the percentage of particle size change after incubation in the phosphate buffer (pH 7.4). Weight amount of microparticles with similar diameter were chosen. Diameter of the beads was measured before and after incubating in phosphate

buffer (pH 7.4) for 12 hrs under optical microscope. The swelling percent was calculated as follow:

$$\text{Swelling ratio\%} = \frac{\text{Diameter in swollen state} - \text{Diameter in dry state}}{\text{Diameter in dry state}} \times 100$$

Compatibility study was performed by Infrared Spectroscopy and the antigen integrity was studied by SDS PAGE and ELISA

In vitro release profile: The 200 mg of the Tetanus vaccines micro particles were taken in a 250ml conical flask. To this 50ml of phosphate buffer (pH7.4) was added. The flask was kept in the shake cum incubator. The shaker was adjusted to 80 horizontal strokes per minute at 37 ^{0}C. From this 1ml of solution was taken in test tube and fresh phosphate buffer was added immediately in the flask. This was repeated at various pre determined time intervals 2hrs, 4hrs, 6hrs, 8hrs, 10hrs, 12hrs, 14hrs, 16hrs, 18hrs, 20hrs. The collected samples were centrifuged and supernatant solution analyzed by Bronsted Lowry's method.

Immunoglobulin titer: Bleeding of immunized Wister rats done by Retro- orbital plexus by using a capillary tube. The samples were pooled and serum was separated. The serum sampled was analyzed by Enzyme linked Immunosorbent assay. The potency of Tetanus vaccine encapsulated starch microparticles may be tested by measuring the specific antibody level was done by Immunoprecipitation assay. The immunoglobulin titer is found by Immune precipitation assay with Nephelometric end point detection.

RESULTS : The polymer was prepared in different analysis method at different concentration (1%,2%,3%,4%,5%,6%,7%,8% and 9%). At 8% of Potato starch suitable for the microparticles formation for the antigen was release as Shown in Table 1. 0.5% of cross inking concentration shows the smooth particles formation, which is shown in Table 2. Antigen capacity before and after loading shows the size reduction as represented in Table 3 and The characteristic of the microparticle size shows the loading capacity and efficiency and swelling property shown in Table 4

Table: 1 The effect of the polymer concentration

Polymer	Polymer conc.	Stabilizing agent	Conc. of stabilizing agent (%)	Size & shape
Starch	1%	Glutaraldehyde	0.5%	Micro particles are not formed
	2%			
	3%			
	4%			
	5%			
	6%			
	7%			
	8%			Microparticles are formed
	9%			Clumping occurs

Table: 2 The effect of cross linking (stabling agent) concentration

Polymer	Polymer conc.	Stabilizing agent	Conc. of stabilizing agent (%)	Size & shape
Starch	8%	Glutaral-dehyde	0.1%	Micro particles are not formed
			0.2%	Micro particles are not formed
			0.3%	Micro particles formed but unstable
			0.4%	Micro particles are formed but not homogeneous
			0.5%	Smooth particles are formed

Table 3 Effect of loading on micro particle formulation.

Characteristic antigen encapsulated starch micro particles	Size		Comments
	Before loading	After loading	
Typhoid antigen encapsulated starch micro particles	66 µm	15-60 µm	Size reduced, smooth micro particles are formed
Dummy batch	72 µm	18-24 µm	Size reduced, smooth micro particles are formed

Table 4. Physical characteristic of prepared microparticles.

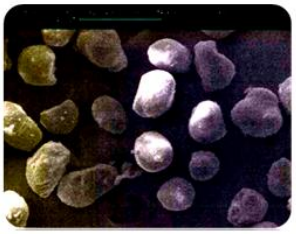
Batch	Particle size (µm)	Loading capacity (%)	Loading efficiency (%)	Swelling ratio
Dummy microparticles	36.35 ± 0.088	-	-	5.43±0.09
Antigen loaded microparticles	39.50 ± 0.03	50.21 ± 0.32%	80.23± 1.5%	2.75 ± 0.16

Values are expressed as mean ± SD (n= 5).

DISCUSSION: The loading of antigen in to the microparticles and the size of the microparticles were greatly influenced by stabilizing agent, polymer concentration by differential analysis (Table 1 & 2).The antigen loading capacity refers the size. The size was reduced and smooth microparticles are formed before and after loading (Table 3) the average size of microparticles was found to be 39.50µm and the particles were having smooth surface the loading capacity was about 30.50% and the loading efficiency as about 80.23%. (Table 4).

In SEM analysis , the antigen encapsulated with potato starch microparticles were found to be irregular size and shape with antigen unloaded microparticles whereas antigen loaded microparticles are homogenous and shown to be more or less spherical geometrical (Fig-1 and 2).

Fig (1) Antigen unloaded micro particle Fig: (2) Antigen loaded micro particles

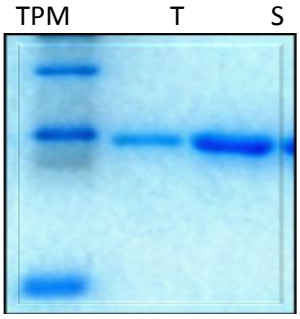


Scanning electron Microscopy shows the particle morphology and density geometrical shape with antigen loaded and unloaded micro particles.

Regarding, stabilities studies, the morphology of tetanus antigen encapsulated starch microparticles were not changed at 4^0C and room temperature but not in 50^0C. The water uptake of the microparticles after 24hrs of incubation leads to bursting of the micro particles. Therefore, it is presumed that water penetrates in to the micro particles, and dissolves the stabilizing agent. According, the size of pores of micro particles was increased and thus the antigen was released which lead to the bursting effect. The integrity of the entrapped antigen was evaluated by SDS PAGE and ELISA. The band intensity shows nature of antigen release from the micro particles and was found that there is no damage occurs during microencapsulation (Fig- 4). The percentage of antigenically active typhoid antigens was found to be 94% by ELISA after entrapment with 8% of starch and 0.5% of stabilizing agent (Glutaraldehyde).

The integrity of the entrapped antigen was evaluated by SDS PAGE and ELISA. The band intensity shows nature of antigen release from the microparticles and was found that there is no damage occurs during microencapsulation (Fig- 3). The percentage of antigenically active tetanus antigens was found to be 91.5% by ELISA after entrapment with 8% starch and 0.5% stabilizing agent (Glutaraldehyde).

Figure (3) SDS PAGE GEL ELECTROPHORESIS



The above picture shows that the TPM – Tetanus marker protein, T test Antigen S- Conventional antigen the band intensity of antigen entrapped potato starch microparticles

In vitro release study : It shows the gradual release of entrapped antigen and peak release was observed on (2 to 20hrs). The release was pulsated for 6 hrs and this was confirmed by the Bronsted Lowry's methods. The percentage of total protein and antigenic active protein release from microparticles was considered to be sustained release of antigenic release (Figure -3&4).

Figure – (4.a &4.b)
Graphical REPRESENTATION OF ir spectropic analysis

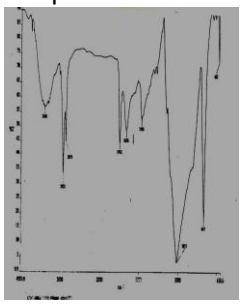


Fig 4.a

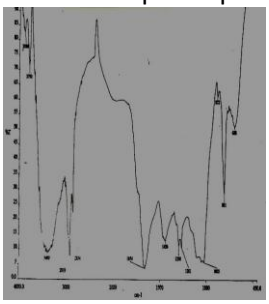


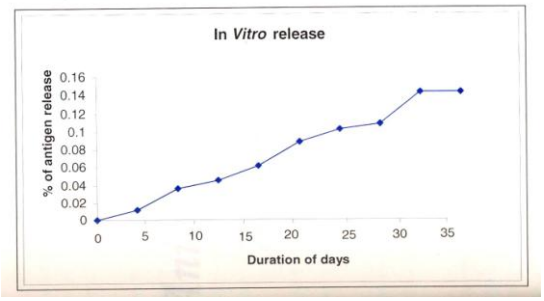
Fig 4.b

Fig 4.a: Antigen unloaded micro particles
Fig 4.b 2.Antigen loaded micro particles

The vibration of the Bond indicates the the antigen absorption at 3000cm-1, 2500cm-1, 4500cm-1-with the unloaded microparticles. The antigen loaded microparticles shows that 1666cm-1, 2500cm-1, 3500cm-1

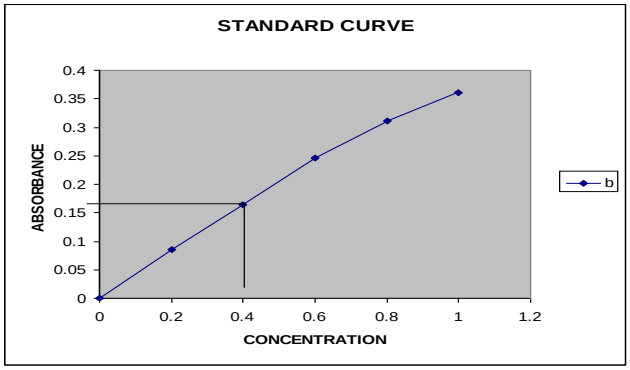
Large amount of Tetanus antigen was incorporated during the microencapsulation process, which increases the loading capacity. (Figure-5&6) Some of earlier researcher reported that the molecular weight of the polymer had a great impact on the formulation of microencapsulation. However, further studies with some modification are needed to get better formulation to release antigenically active tetanus antigen

Figure –(5) In vitro release profile



Each point indicates that the sustain release of the microsphere till 35th day

Figure (6) STANDARD CURVE FOR THE ANTIGEN RELEASE



In vivo immune response: The antigen is release slowly from the site of injection. The small micro particles can be directly taken up in to macrophages by phagocytes However, larger micro particles need to undergo biodegradation before phagocytosis can occur; in this stage micro particles are covered by one or several layers of macrophages as a consequence of the wound healing response to injected particles. In this research work, the size of the Tetanus antigen loaded starch micro particles was 39.50 µm; consequently the vaccine loaded large micro particles undergo degradation and antigen release from the micro particles. From the Figure (7,8&9) the Immunoglobulin titer values shows that releases of the antigen with IgG increase in level with correspond to IgA and Ig E and IgM .

Figure (7) Immunoglobulin Titer

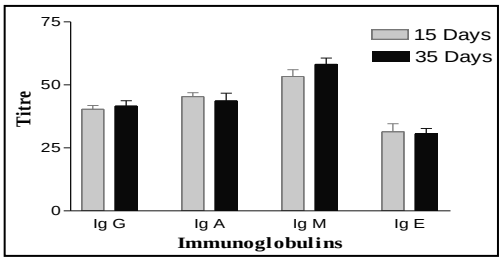


Figure (8) Immunoglobulin Titer
IgG, Ig A, Ig M, Ig E, Anti T-Ag. of potato starch

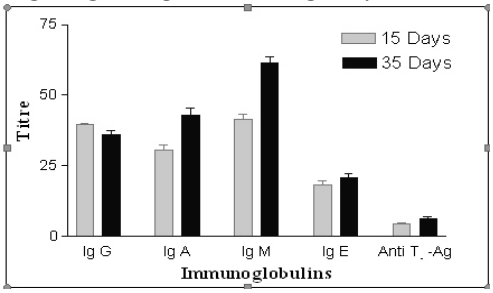
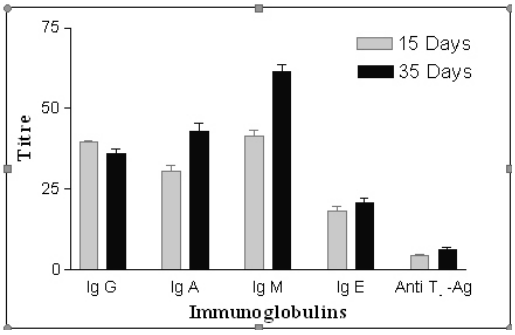


Figure (9) Immunoglobulin Titer
(Conventional Tetanus Vaccine)



The Anti Tetanus antigen is increased in the titre values (Figure 8&9) whtn compare to conventional vaccines the Potato starch encapsulated tetanus antigen shows Increase immune response

CONCLUSION: Adjuvant aimed to increasing the immunogenicity of recombinant antigens remains a focus in vaccine development. Worldwide, there is currently considerable interest in the development of biodegradable microspheres for the controlled release of vaccines, since the major disadvantages of several currently available vaccine is the need for repeated administration concerning, the potential ability and development of the single dose vaccine for the Tetanus antigen, by using natural biodegradable polymer due to its cost in expensive, rapid releasing rate in controlled manner, like water soluble polysaccharide derivatives such as Starch. The immunogenicity status shows the feasibility of covering the present three injection schedule for Tetanus antigen in to a single shot therapy.

The polymeric microparticles of Starch were successfully formulated by using encapsulation technique. The size of the vaccine loaded microparticles was formulated.The immunogenicity of Tetanus vaccine loaded Potato starch microparticles was determined by antibody induction method. This shows the good immune response till 35th day. However it is clear that more detailed investigation are necessary to clarify the effect of matrix polymer on antigen stability and enhanced immunogenicity during microspheres preparation and antigen releasing procedures and the effect of immunization of animals with microspheres formulation.

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